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APPROV #

Our Ref.: 6580-085/MG

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Assistant Commissioner for Patents
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U.S.A. 20231

Dear Sir:

PROVISIONAL APPLICATION UNDER 37 C.F.R. § 1.53(b)(2)

This is a request for filing a Provisional application under 37 C.F.R. § 1.53(b)(2) entitled **PRODUCTION OF RECOMBINANT PROTEINS IN EGGS** by the following inventor(s):

Full Name of Inventor	**Family Name** ETCHES	**First Given Name** Robert	**Second Given Name** J.
Residence & Citizenship	**City** Guelph	**State or Foreign Country** Ontario	**Country of Citizenship** Canada
Post Office Address	**Post Office Address** c/o University of Guelph	**City** Guelph	**State & Zip Code/Country** Ontario, N1G 2W1 Canada
Full Name of Inventor	**Family Name** MOHAMMED	**First Given Name** Mansoor	**Second Given Name**
Residence & Citizenship	**City** Guelph	**State or Foreign Country** Ontario	**Country of Citizenship** Canada
Post Office Address	**Post Office Address** c/o University of Guelph	**City** Guelph	**State & Zip Code/Country** Ontario, N1G 2W1 Canada
Full Name of Inventor	**Family Name** MORRISON	**First Given Name** Sherie	**Second Given Name**
Residence & Citizenship	**City** Los Angeles	**State or Foreign Country** California	**Country of Citizenship** U.S.A.
Post Office Address	**Post Office Address** c/o University of California, Los Angeles	**City** Los Angeles	**State & Zip Code/Country** California, 90095-1406, U.S.A.

1. __X__ Enclosed is the Provisional application as follows: __24__ pages of specification, __18__ claims, __1__ page Abstract and ____ sheets of drawings.

2. _____ Enclosed is a Verified Statement that this filing is by a small entity (37 C.F.R. 1.9, 1.27, 1.28).

3. __X__ Payment of Provisional filing fee under 37 C.F.R. § 1.16(k):

   __X__ Attached is a cheque in the amount of $150.00. cheque # 59
   ____ Please charge the filing fee of $150.00 from our Deposit Account No. 02-2095. The letter is being filed in triplicate.
   ____ PAYMENT OF THE FILING FEE IS BEING DEFERRED.

4. __X__ The Commissioner is hereby authorized to charge any additional fees as set forth in 37 C.F.R. §§ 1.16 to 1.18 which may be required by this paper or credit any overpayment to Account No. 02-2095.

5. ____ Enclosed is an Assignment of the invention to _____. Recordation Form Cover Sheet and a cheque for $40 to cover the Recordation Fee.

6. ____ Also enclosed:

7. ____ The invention was made by the following agency of the United States Government or under a contract with the following agency of the United States Government: _____.

8. __X__ Address all future communications to the Attention of Micheline Gravelle at the address below.

Respectfully submitted,

August 21, 1997
Date

M. Gravelle
Micheline Gravelle
Registration No. 40,261

BERESKIN & PARR
Box 401
40 King Street West
Toronto, Ontario
Canada M5H 3Y2
Telephone: 416-364-7311

B&P File No. 6580-085/MG

BERESKIN & PARR **UNITED STATES**

Title: Production of Recombinant Proteins in Eggs
Inventors: Robert J. Etches, Mansoor Mohammed and Sherie Morrison

B&P File No. 6580-085/MG

Title: Production of Recombinant Proteins in Eggs

FIELD OF THE INVENTION

The invention relates to the production of recombinant proteins in eggs.

BACKGROUND OF THE INVENTION

Biotechnology has allowed the improved production of proteins that have many important medical applications such as the diagnosis and therapy of disease. Unfortunately many of the existing methods for producing recombinant proteins are prohibitive due to the high cost for the large scale production and purification of the proteins.

Antibody molecules are one type of protein that have been prepared using biotechnology. Antibodies (or immunoglobulins) are highly specific tools useful in both the therapy and diagnosis of various diseases and pathogens. Briefly, an intact antibody or immunoglobulin molecule consists of 2 heavy (H) and 2 light chains (L), each having a constant region at the carboxy terminus and a variable region at the amino terminus. Several constant region isotypes have been identified for human immunoglobulins, two for the light chain (kappa and lambda) and five for the heavy chain (alpha, gamma, delta, epsilon and mu). As the name denotes, the sequence of the variable regions varies in each immunoglobulin molecule. The variable region contains the antigen binding site and thus determines the antigen specificity of the immunoglobulin molecule.

When immunizing humans, it is desirable to use human antibodies in order to avoid an immune reaction against the immunizing antibodies. However, due to practical and ethical considerations it has not been possible to prepare large quantities of human antibodies from a human source. Although human Igs derived from serum or breast milk have demonstrated efficacy, the high cost and limited supply of human products preclude their widespread application. In order to decrease the immune response against non-human antibody preparations, chimeric or humanized antibodies have been prepared.

Chimeric antibodies are genetically engineered so that the constant region of the antibody is derived from a human antibody and the variable region is derived from the immunized, generally non-human, host. The variable region is usually derived from an antibody isolated from a rodent that has been immunized with the desired antigen.

One area where antibodies are useful is in the treatment of enteric infections. Enteric infections resulting in diarrhea, dysentery or enteric fever constitute a huge public health problem with more than a billion episodes of disease and several million deaths annually in the developing countries. Rotaviruses are one major cause of infectious gastroenteritis in infants and young children in both developed and developing countries. Enterotoxigenic *Escherichia coli* (ETEC) are another major causative agent and result in over 600 million cases of diarrhea worldwide annually. ETEC disease is initiated by consumption of contaminated food or water. Bacteria transit to and colonize the upper small bowel and produce heat stable and/or heat labile enterotoxins. Both types of pathogen should be susceptible to treatment of antibodies targeted to the mucosal surface.

SUMMARY OF THE INVENTION

The present invention relates to the preparation of a recombinant protein in an egg.

The present inventors have shown that recombinant proteins, such as antibodies, can be expressed in egg laying animals and transported to the egg. The recombinant protein may be introduced into the animal using an expression vector that contains a DNA sequence encoding the desired protein and necessary regulatory regions to provide for expression of the recombinant protein. In addition, the expression vector will contain a second DNA sequence which can target or deliver the protein to the egg of an egg-laying animal.

Accordingly, in one aspect, the present invention relates to an expression vector for delivering a recombinant protein to an egg comprising (i) a first DNA sequence encoding the recombinant protein (ii) a regulatory region sufficient to provide for expression of the protein

and (iii) a second DNA sequence which can facilitate the delivery of the protein to an egg of an animal.

The second DNA may encode a regulatory region derived from an egg specific gene that can target the expression of the recombinant protein to the egg. Alternatively, the second DNA sequence may encode a protein or peptide that can bind to a receptor on the egg resulting in the uptake of the protein into the egg. In one embodiment, the second DNA sequence encodes a portion of an immunoglobulin molecule sufficient to bind to the egg and result in the uptake of the recombinant protein. In a specific embodiment, the second DNA sequence is derived from an immunoglobulin constant region. In fact, the present inventors have surprisingly found that the constant region from a human immunoglobulin can bind to an avian oocyte and be internalized into the yolk.

In one embodiment, the present invention relates to the preparation of a recombinant antibody molecule in a fowl egg. Fowl eggs contain receptors for antibody molecules. Consequently, the antibody will be delivered to the egg without the need for adding an additional sequence to facilitate the delivery. In a preferred embodiment, the present invention relates to the preparation of humanized antibodies in chicken eggs.

The term "humanized antibody" as used herein means an immunoglobulin or antibody molecule that contains a human constant region. The humanized antibody may be chimeric and contain the variable region from a non-human such as a chicken, mouse, etc. The antibody may also be non-chimeric and contain human variable regions. The terms "antibody" and "immunoglobulin" may be used interchangeably throughout the application.

Accordingly, the present invention provides, an expression vector for delivering a recombinant antibody to an egg comprising (i) a first DNA sequence encoding an immunoglobulin constant region (ii) a second DNA sequence encoding an immunoglobulin variable region and (iii) a regulatory region sufficient to provide for expression of the

antibody. Preferably, the constant region is derived from a human immunoglobulin.

The expression vector may be introduced directly into the egg-laying animal where the recombinant protein will be expressed and delivered to the egg. Accordingly, the present invention provides a method of preparing a recombinant protein in an egg comprising:

a) introducing an expression vector into an egg-laying animal, wherein the expression vector comprises (i) a DNA sequence encoding the recombinant protein (ii) a regulatory region sufficient to provide for expression of the protein and (iii) A DNA sequence which facilitates the delivery of the protein to an egg of the animal;

b) allowing the animal to lay an egg;

c) obtaining the egg containing the recombinant protein; and optionally

d) isolating the recombinant protein from the egg.

In one embodiment, the present invention provides a method of preparing a recombinant antibody in an egg comprising:

a) introducing an expression vector into an egg-laying animal, wherein the expression vector comprises (i) a first DNA sequence encoding an immunoglobulin constant region (ii) a second DNA sequence encoding an immunoglobulin variable region and (iii) a regulatory region sufficient to provide for expression of the antibody;

b) allowing the animal to lay an egg;

c) obtaining the egg containing the recombinant antibody; and optionally

d) isolating the recombinant protein from the egg.

As an alternative to introducing the expression vector directly into the animal, the vector may be transfected in culture into a host cell. The host cell can be injected into the egg-laying animal where the protein will be secreted. The host cell is preferably of the same species as the egg-laying animal. In a specific embodiment, the host cell is an avian cell line. When the recombinant protein is an antibody, the avian

cell line is preferably a lymphoid cell line, more preferably an immortalized B cell line such as DT40 or a v-rel transformed B cell line.

Accordingly, in another embodiment, the present invention relates to a method of preparing a recombinant protein in an egg comprising:

a) introducing a transformed avian cell line that secretes a recombinant protein into an egg-laying animal wherein the avian cell line has been transformed with an expression vector comprising (i) a first DNA sequence encoding the recombinant protein (ii) a regulatory region sufficient to provide for expression of the protein and (iii) a second DNA sequence which can effect the delivery of the protein to an egg of an animal;

b) allowing the animal to lay an egg;

c) obtaining the egg containing the recombinant protein; and optionally

d) isolating the recombinant protein from the egg.

In a preferred embodiment, the present invention relates to a method of preparing a recombinant antibody in a fowl egg comprising:

a) introducing a transformed avian cell line that secretes a recombinant antibody into an egg laying fowl wherein the avian cell line has been transformed with an expression vector comprising (i) a first DNA sequence encoding an immunoglobulin constant region (ii) a second DNA sequence encoding an immunoglobulin variable region and (iii) a regulatory region sufficient to provide for expression of the antibody;

b) allowing the fowl to lay an egg;

c) obtaining the egg containing the recombinant antibody; and optionally

d) isolating the recombinant antibody from the egg.

Preferably, the recombinant antibody is a humanized antibody and the fowl is a chicken. The present inventors have demonstrated for the first time that humanized antibodies (containing

non-chicken constant regions) can be transported to and isolated from the chicken egg.

In a another aspect, the recombinant protein may be delivered to the egg by preparing a transgenic egg-laying animal that expresses the recombinant protein in conjunction with a protein or peptide that delivers the protein to the egg. Preferably the animal is a fowl, the recombinant protein is an antibody such as a humanized antibody.

In another aspect, the present invention relates to a method of preparing an egg that is free of a particular pathogen comprising:

(a) introducing an antibody specific for the pathogen into an egg laying animal; and

(b) allowing the animal to lay an egg wherein the egg is substantially free of the pathogen.

In another aspect, the present invention relates to an egg preparation containing a recombinant protein as well as all uses of the egg preparation for example in the diagnosis, prevention and treatment of various diseases. The egg preparation can be used directly or the recombinant protein can be further isolated and purified from the egg.

In one embodiment, the antibodies are useful in the prevention and treatment of enteric infections.

Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples while indicating preferred embodiments of the invention are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

DETAILED DESCRIPTION OF THE INVENTION

Broadly stated, the present invention relates to the preparation of recombinant proteins in eggs. The recombinant protein may be any useful protein and can include antibodies, cytokines,

hormones, enzymes, antigens for vaccines and diagnostic applications, and therapeutic peptides.

The eggs may be from any egg-laying animal including birds, amphibians, reptiles and fish. Preferably, the eggs are from a fowl such as chicken, turkey or duck. The use of eggs as a source of recombinant proteins offers considerable advantages including compatibility with modern animal protection regulations, cheapness, convenience, sterility and the availability of technology for fractionation of egg yolk and isolation of proteins such as antibodies. Since a single egg can yield approximately 100 mg of antibody, a single hen laying 250 eggs per year can produce 25 g of Ig and a small flock of 10,000 hens can produce 25 kg of immunoglobulin annually. Eggs can be stored at room temperature for several weeks.

Expression vectors

As hereinbefore mentioned, the present inventors have shown that recombinant proteins, such as antibodies, can be expressed in egg laying animals and transported to the egg. The recombinant protein may be introduced into the animal using an expression vector that contains a DNA sequence encoding the desired protein and necessary regulatory regions to provide for expression of the recombinant protein. In addition, the expression vector will contain a second DNA sequence which can target or deliver the protein to the egg of an egg-laying animal.

Accordingly, in one aspect, the present invention relates to an expression vector for delivering a recombinant protein to an egg comprising (i) a first DNA sequence encoding the recombinant protein (ii) a regulatory region sufficient to provide for expression of the protein and (iii) a second DNA sequence which facilitates the delivery of the protein to an egg of an animal.

The second DNA may encode a regulatory region derived from an egg specific gene that can target the expression of the recombinant protein to the egg. Alternatively, the second DNA sequence may encode a protein or peptide that can bind to a receptor on the egg

resulting in the uptake of the protein into the egg. In one embodiment, the second DNA sequence encodes a portion of an immunoglobulin molecule sufficient to bind to the egg and result in the uptake of the recombinant protein. In a specific embodiment, the DNA sequence is derived from an immunoglobulin constant region. In fact, the present inventors have surprisingly found that the constant region from a human immunoglobulin can bind to an avian oocyte and be internalized into the yolk. Other proteins or peptides that bind to an egg- specific receptor and can be used in the present invention include vitellogenin and apolipoprotein B.

The expression vector of the present invention may be a viral or a non-viral vector and can be constructed using techniques known in the art. Phagemids are an example of a useful vectors because they can be used either as plasmids or as bacteriophage vectors. Examples of other vectors include viruses such as bacteriophages, baculoviruses and retroviruses, DNA viruses, liposomes and other recombination vectors. The vectors can also contain elements for use in eucaryotic host systems, preferably an avian host system.

In a specific embodiment, the present invention relates to the preparation of a recombinant antibody in an egg. In such an embodiment, the above described second DNA sequence which delivers the recombinant protein to the egg is naturally present in the immunoblogulin constant region.

Accordingly, the present invention provides an expression vector for delivering a recombinant antibody to an egg comprising (i) a first DNA sequence encoding an immunoglobulin variable region (ii) a second DNA sequence encoding an immunoglobulin constant region and (iii) a regulatory region sufficient to provide for expression of the antibody.

In a preferred embodiment, the present invention relates to the preparation of humanized antibodies in chicken eggs. As defined herein, the humanized antibodies contain at least a human constant region. The constant region can be selected from any of the known

constant regions including the kappa and lambda light chains and the alpha, gamma, delta, epsilon and mu heavy chain genes. The variable region may be human or non-human such as avian, ovine, murine or bovine. When the variable and constant regions are from different species then the antibody is termed a "chimeric antibody". Chimeric antibodies may be prepared using techniques known in the art such as described in Morrison et al. Proc. Natl. Acad. Sci. 81:6851-6859, 1984 which is incorporated herein by reference.

The variable region may have specificity for a desired antigen. The desired antigen may be selected from bacteria, viruses, toxins, allergens as well as disease specific antigens including tumor associated antigens. A variable region gene encoding a variable region with a desired antigen specificity may be obtained from a hybridoma producing a monoclonal antibody with the desired antigen specificity. A hybridoma producing an antibody with the desired specificity may also be prepared using techniques known in the art. Briefly, an animal (such as a chicken, mouse or rabbit) may be immunized with the desired antigen and lymphocytes producing the antibodies may be obtained. The lymphocytes may be immortalized by fusion with immortal cells such as myeloma cells to prepare a hybridoma. A hybridoma producing the desired antibody may be selected using techniques known in the art (see for example Kohler and Milstein, Nature 256:495-497, 1975). The desired variable region gene can then be isolated from the hybridoma using known techniques such as polymerase chain reaction (PCR).

Bifunctional antibodies may also be prepared which contain two different variable regions with two different antigen specificities.

The DNA sequences encoding the human constant region can be obtained from available sources or can be isolated from a hybridoma cell line producing an antibody with a human constant region using techniques described above.

Recombinant expression vectors containing the DNA sequence encoding a human constant region and the DNA sequence encoding the desired variable region may be prepared. The vectors will

additionally include expression control or regulatory sequences such as a promoter, an enhancer and termination sequences. Preferred regulatory sequences are derived from immunoglobulin genes but additional regulatory regions such as those derived from viruses may be useful. The vector can be selected from a variety of vectors including plasmids, viruses, retroviruses, and adenoviruses.

Pre-formed expression vectors may also be prepared that contain the DNA sequence encoding the constant region and the necessary regulatory sequences. A desired variable region DNA sequence can be inserted into the preformed vector in order to prepare an antibody with a desired antigen specificity. The pre-formed vector thus facilitates the preparation of the desired humanized antibody.

Delivery and targeting to egg

The recombinant proteins of the present invention may be introduced into the animal using techniques known in the art.

In one embodiment, the expression vector is introduced directly into the animal.

Accordingly, the present invention provides a method of preparing a recombinant protein in an egg comprising:

a) introducing an expression vector into an egg-laying animal, wherein the expression vector comprises (i) a first DNA sequence encoding the recombinant protein (ii) a regulatory region sufficient to provide for expression of the protein and (iii) a second DNA sequence which can facilitate the delivery of the protein to an egg of an animal;

b) allowing the animal to lay an egg;

c) obtaining the egg containing the recombinant protein; and optionally

d) isolating the recombinant protein from the egg.

The vectors can be introduced into the cells or tissues of the egg-laying animal by any one of a variety of known methods within the art. Such methods can be found generally described in Sambrook et al., *Molecular Cloning: A Laboratory Manual,* Cold Springs Harbor

Laboratory, New York (1989, 1992), in Ausubel et al., *Current Protocols in Molecular Biology*, John Wiley and Sons, Baltimore, Maryland (1989), Chang et al., *Somatic Gene Therapy*, CRC Press, Ann Arbor, MI (1995), Vega et al., *Gene Targeting*, CRC Press, Ann Arbor, MI (1995), *Vectors: A Survey of Molecular Cloning Vectors and Their Uses*, Butterworths, Boston MA (1988) and Gilboa et al (1986) and include, for example, stable or transient transfection, lipofection, electroporation and infection with recombinant viral vectors.

Introduction of the vectors by infection offers several advantages. Higher efficiency can be obtained due to their infectious nature. Moreover, viruses are very specialized and typically infect and propagate in specific cell types. Thus, their natural specificity can be used to target the vectors to specific cell types *in vivo* or within a tissue or mixed culture of cells. Viral vectors can also be modified with specific receptors or ligands to alter target specificity through receptor mediated events.

Additional features can be added to the vector to ensure its safety and/or enhance its efficacy. Such features include, for example, markers that can be used to negatively select against cells infected with the recombinant virus. An example of such a negative selection marker is the TK gene described above that confers sensitivity to the anti-viral gancyclovir. Negative selection is therefore a means by which infection can be controlled because it provides inducible suicide through the addition of antibiotic. Such protection ensures that if, for example, mutations arise that produce altered forms of the viral vector or sequence, cellular transformation will not occur. Features that limit expression to particular cell types can also be included. Such features include, for example, promoter and regulatory elements that are specific for the desired cell type.

Recombinant viral vectors are another example of vectors useful for *in vivo* introduction of a desired nucleic acid because they offer advantages such as lateral infection and targeting specificity. Lateral infection is inherent in the life cycle of, for example, retrovirus and is the

process by which a single infected cell produces many progeny virions that bud off and infect neighboring cells. The result is that a large area becomes rapidly infected, most of which was not initially infected by the original viral particles. This is in contrast to vertical-type of infection in which the infectious agent spreads only through daughter progeny. Viral vectors can also be produced that are unable to spread laterally. This characteristic can be useful if the desired purpose is to introduce a specified gene into only a localized number of targeted cells.

Retroviral vectors can be constructed to function either as infectious particles or to undergo only a single initial round of infection. In the former case, the genome of the virus is modified so that it maintains all the necessary genes, regulatory sequences and packaging signals to synthesize new viral proteins and RNA. Once these molecules are synthesized, the host cell packages the RNA into new viral particles which are capable of undergoing further rounds of infection. The vector's genome is also engineered to encode and express the desired recombinant gene. In the case of non-infectious viral vectors, the vector genome is usually mutated to destroy the viral packaging signal that is required to encapsulate the RNA into viral particles. Without such a signal, any particles that are formed will not contain a genome and therefore cannot proceed through subsequent rounds of infection. The specific type of vector will depend upon the intended application. The actual vectors are also known and readily available within the art or can be constructed by one skilled in the art using well-known methodology.

Transfection vehicles such as liposomes can also be used to introduce the non-viral vectors described above into recipient cells within the inoculated area. Such transfection vehicles are known by one skilled within the art.

In a second embodiment, the recombinant protein may be delivered to the egg by introducing a host cell that has been transformed with an expression vector of the present invention into the egg-laying animal. The transformed cell line will secrete the recombinant protein which will be transported to the egg. Preferably the host cell is an avian

cell line, specifically a pluripotent or multipotent embryonic cell line, a cell line committed to the germ line or any cell line that can contribute to somatic tissues and the germ line.

Accordingly, the present invention provides a method for preparing a recombinant protein in an egg comprising

a) introducing a transformed avian cell line that secretes a recombinant protein into an egg-laying animal wherein the avian cell line has been transformed with an expression vector comprising (i) a first DNA sequence encoding the recombinant protein (ii) a regulatory region sufficient to provide for expression of the protein and (iii) a second DNA sequence which facilitates the delivery of the protein to an egg of an animal;

b) allowing the animal to lay an egg;

c) obtaining the egg containing the recombinant protein; and, optionally

d) isolating the recombinant protein from the egg.

In a specific embodiment, the avian cell line secretes a recombinant antibody, preferably a humanized antibody. The avian cell line may be injected into laying hens. The antibodies will be produced *in vivo* in the hen and will be delivered to and can be obtained from the yolk of the eggs.

Accordingly, the present invention provides a method of preparing a recombinant antibody in a fowl egg comprising:

a) introducing a transformed avian cell line that secretes a recombinant antibody into an egg laying fowl wherein the avian cell line has been transformed with an expression vector comprising (i) a first DNA sequence encoding an immunoglobulin constant region (ii) a second DNA sequence encoding an immunoglobulin variable region and (iii) a regulatory region sufficient to provide for expression of the antibody;

b) allowing the fowl to lay an egg;

c) obtaining the egg containing the recombinant antibody; and optionally

d) isolating the recombinant antibody from the egg.

In a third embodiment, the recombinant proteins of the present invention may be prepared in an egg laying animal by preparing a transgenic animal that secretes the recombinant protein. In a preferred embodiment, a recombinant antibody may be prepared in a fowl by preparing a transgenic fowl that secretes the antibody, preferably a humanized antibody. To prepare a transgenic fowl, an expression vector containing the DNA encoding the human constant region and desired variable region can be inserted into fowl embryos using techniques known in the art including microinjection, electroporation, sperm transfection, liposome fusion and microprojectile bombardment. The embryos containing the expression vector are then transferred to a surrogate shell. The chicks carrying the transgene can be grown to sexual maturity and the presence of the chimeric antibody can be analyzed in the eggs of the mature chicken.

The present invention can also be used to prepare pathogen free eggs. For example, an antibody specific for a particular pathogen can be produced in an egg laying animal and transported to the egg where it will neutralize the particular pathogen. In one embodiment, the antibody may be an anti-salmonella antibody and can be used to prepare salmonella free eggs.

Consequently, in another aspect, the present invention relates to the preparation of an egg that is free of a particular pathogen comprising:

(a) introducing an antibody specific for the pathogen into an egg laying animal; and

(b) allowing the animal to lay an egg wherein the egg is substantially free of the pathogen.

Egg Preparations

The present invention also includes the eggs containing the recombinant proteins as well as the use of the eggs in all applications. Since eggs are an edible food source, the recombinant proteins do not have to be isolated of purified from the egg. The eggs containing the

recombinant protein can be consumed directly or they can be cooked or incorporated into recipes (such as omelets, shakes, baked goods) prior to consumption.

If desired, the recombinant protein can be isolated from the egg and incorporated into a pharmaceutical formulation prior to administration. For example, the humanized antibodies can be removed from the chicken egg using techniques known in the art (see for example USP 5,420,253). The antibodies are generally contained in the yolk of the egg which is separated from the rest of the egg in order to obtain the antibodies. The yolk preparation containing the antibodies or other recombinant protein may be lyophilized for storage. The lyophilized preparation may be reconstituted when ready for use.

The antibodies can be used to treat or detect various diseases or pathogens depending on the specificity of the variable region. For the treatment of disease, the antibodies may be administered alone, conjugated or in combination with other compounds. The antibodies may be conjugated to a toxin in order to facilitate the destruction of the diseased or infected cells once the antibody binds to it. Such conjugated antibodies are known as immunotoxins and may be prepared using techniques known in the art (Thorpe et al., Monoclonal Antibodies in Clinical Medicine, Academic Press, p. 168-190, 1982).

The recombinant proteins or antibodies may be prepared in a pharmaceutical composition suitable for administration *in vivo*. The pharmaceutical composition may contain the protein or antibody in a biologically compatible carrier or diluent or in a carrier system such as liposomes. The protein or antibody composition may be administered in a convenient matter such as by injection, oral administration, inhalation, transdermal application or rectal administration. Depending on the route of administration, the active compound may be coated on to a material to protect the compound from the action of enzymes, acids or other natural conditions which may inactivate the antibody. The composition will contain a therapeutically effective amount of the

protein or antibody and will be provided at dosages and periods of time necessary to achieve the desired results.

The antibodies may be used for the *in vivo* or *in vitro* diagnosis or detection of disease. For *in vivo* diagnostics, the antibodies will be prepared in suitable pharmaceutical formulations as discussed above. The antibodies are also generally labelled with a detectable marker to allow their detection. The detectable markers which may be used include various enzymes, fluorescent materials, luminescent materials and radioactive materials. Examples of suitable enzymes include horseradish peroxidase, biotin, alkaline phosphatase, ß-galactosidase, or acetylcholinesterase; examples of suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin; an example of a luminescent material includes luminol; and examples of suitable radioactive material include S-35, Cu-64, Ga-67, Zr-89, Ru-97, Tc-99m, Rh-105, Pd-109, In-111, I-123, I-125, I131, Re-186, Au-198, Au-199, Pb-203, At-211, Pb-212 and Bi-212. The antibodies may also be labelled or conjugated to one partner of a ligand binding pair. Representative examples include avidin-biotin and riboflavin-riboflavin binding protein. Methods for conjugating or labelling the antibodies discussed above with the representative labels set forth above may be readily

The antibodies may also be used to detect disease or pathogens *in vitro* using techniques known in the art. The methods rely on the binding interaction between the antibodies an antigenic determinant of a protein specific to the pathogen or disease. Examples of such methods are radioimmunoassays, enzyme immunoassays (e.g. ELISA), immunofluorescence, immunoprecipitation, latex agglutination, hemagglutination, and histochemical tests. such as enzyme-linked immunosorbant assay (ELISA), and western blotting.

The antibodies of the present invention may be used to treat enteric infections such as rotavirus infection and enterotoxigenic *Escherichia coli* (ETEC) as these are the major causitive agents of disease

in newborns and children. Antibodies may be prepared that contain variable regions that are specific for these pathogens or parts of the pathogens.

The following non-limiting examples are illustrative of the present invention:

EXAMPLES

Example 1

Preparation Of Humanized Antibodies In Chicken Cell Lines

Material And Methods

Culture and transfection of cell lines

A chicken B lymphoblastoid cell line, DT40, derived from Hyline SC™ chickens (Hyline International, Dallas Center, Iowa) was obtained from Dr. Craig Thompson and used to establish transfected cell lines producing human/mouse chimeric antibodies. Cells were maintained in culture at 1-10 x 10^6 cells/ml in IMDM™ (Gibco BRL) containing 8%(v/v) Bovine Calf Serum (BCS and 2% (v/v) Chicken Serum (CS). Cells (1 x 10^7) were transfected with 20ug each of linearized heavy chain (chimeric anti-dansyl gamma 1) and light chain (chimeric anti-dansyl light chain with human kappa) by means of electroporation using a Bio-Rad electroporator under optimized electroporation conditions (200V, 960uF and 1000msec pulse). Cells were maintained for two days in the above culture media in 96-well micro-titer dishes (2.5 x 10^4 cells/well) after which selection medium containing 3 ug/ml mycophenolic acid, 7.5 ug/ml hypoxanthine and 125 ug/ml xanthine was added. Surviving colonies were screened by enzyme-linked immunosorbent assay (ELISA) using dansyl-BSA coated microtiter plates and alkaline phosphatase linked anti-human kappa as the detecting reagents. Strongly positive colonies were then moved into larger dishes for further characterization. Cells from these expanded colonies were labeled by overnight growth in the presence of ^{35}S-methionine. Following overnight growth, culture supernatant and cytoplasmic lysates were prepared and the contents immunoprecipitated using rabbit anti-human Ig and Staph A (IgSorb). Samples were analyzed on 5%

polyacrylamide gels without reduction and on 12% gels following reduction. The position of the bands were determined by autoradiography. Cells from colonies that produced the desired chimeric antibodies were then maintained in culture medium at 1-10 x 10^6 cells/ml.

Production of tumors in Hyline SC hens

A transfected DT40 cell line, TAOD 7.4, producing chimeric human anti-dansyl gamma3 was maintained in culture at a concentration of 1 x 10^6 cells/ml in Opti-MEM I™ (Gibco BRL, Burlington) containing 10% Fetal Bovine Serum (FBS). Cells were collected by centrifugation at 300 x g for 5 minutes and the culture medium removed. Cells were resuspended at a concentration of 5 x 10^7 cells/ml in Dulbecco's phosphate buffered saline (PBS, Gibco BRL, Burlington, Ontario). A total of 5 x 10^6 cells in 100 ul of PBS was injected subcutaneously into the region between the thigh and body wall of Hyline SC™ hens and tumor development was monitored on a daily basis. Hens were weighed prior to injection and then twice weekly to monitor any fluctuations in weight.

Purification and analysis of yolk antibodies

Eggs were collected from injected hens daily. The yolk was separated from the albumin and antibodies purified from the yolk by means of a gamma Yolk™ preparation kit (Pharmacia Biotech, Morgan Blvd., Quebec). Purified yolk antibody was resuspended in carbonate buffer, pH 9.6, and analyzed for the presence of chimeric human anti-dansyl gamma3 by means of a sandwich antibody ELISA. Immulon™ 96-well microtitre plates were coated overnight at 4°C with 50 ul of a 5ug/ml solution of goat anti-human IgG (H+L) (Jackson Immunoresearch, West Grove, PA, USA) in carbonate buffer, pH 9.6. After overnight incubation the coating mixture was discarded and the plates washed three times with PBS. Plates were then blocked for 1 hr at room temperature by adding 100ul of blocking buffer (PBS containing 3% (w/v) Bovine Serum Albumin). The blocking buffer was then discarded and the plates washed three times with PBS. Individual yolk antibody preparations or serially

diluted standards (Cromopure Human IgG, Jackson Immunoresearch, West Grove PA, USA) (50 ul) were dispensed into each well and the plate incubated overnight at 4°C. After overnight incubation, the test solutions were discarded and the plates washed three times with PBS. Horseradish peroxidase-conjugated goat anti-human IgG (H+L) (Jackson Immunoresearch, West Grove PA, USA) (50 UL), at a concentration of 125 ng/ml in blocking buffer, was added to the wells of the plates and incubated at room temperature for 2 hours. The peroxidase-conjugate was then discarded and the plates washed three times with PBS. Horseradish-peroxidase substrate (ammonium acetate-citric acid buffer (pH 5.0) containing 0.05% (w/v) o-phenylenediamine dihyrochloride and 0.05% (v/v) 30% hydrogen peroxide) was then added to each well and the plates incubated in the dark at room temperature for 30 min. Sulphuric acid (50 ul of a 5M solution) was added to each well and the plates were shaken gently on a table top shaker for 10 min. Colour development was then assessed using a Titertek Multiskan™ PLUS ELISA plate reader with a 492nm filter.

Results

A typical standard curve for the assay of human immunoglobulins in egg yolk is shown in Table 1. Absorbance in the presence of egg yolk is not different from absorbance from a negative control well containing only buffer indicating that egg yolk does not interfere with the assay. The regression coefficient between absorbance at 492nm and log10 concentrations of human immunoglobulin (hIg) is 0.99, indicating that the equation $y=1.1683x-0.0185$ accurately describes the relationship between absorbance and the concentration of hIg.

The effect of yolk in the assay was further examined by constructing a standard curve in presence of yolk extract. As indicated in the graph below, absorbance was unaltered by the presence of yolk at all concentrations of standard in the assay. Absorbance from extracts of yolk from uninjected hens was equal to absorbance at any standard less than 1.56 ng/ml, indicating that concentrations of human Ig less than 1.56 ng/ml could not be detected.

The concentrations of human immunoglobulin in eggs from hen #9185 (Cage #2) are presented in Table 2. This hen was injected with 5 million cells transfected with human IgG3 (TAOD7.4) on day 1. The tumor remained as a small nodule until day 11, at which time hemorrhaging occurred in the region surrounding the tumor. Deposition in yolk was evident in eggs laid on day 13 and 15 although subsequent eggs containing undetectable amounts of hIg.

Discussion

The ELISA for the detection of human immunoglobulin in egg yolk was demonstrated to be sensitive to 1.56 ng/ml, was specific for human immunoglobulin and was reproducible. The recovery of human immunoglobulin from egg yolk was approximately 15% (data not shown).

The presence of a tumor in hen #9185 indicates that DT40 cells will colonize a host chicken to form a somatic chimera. Examination of the concentrations of hIg in yolk from this hen demonstrates that human immunoglobulins are produced by genetically engineered DT40 cells *in vivo* and sequestered into egg yolk. Since there are approximately 10-15 ml per egg yolk, and the recovery of hIg in the assay was approximately 15%, it is expected that about 625 ng of hIg were deposited in the egg laid by hen #9185 on day 13.

These data provide the rationale for developing a technology for the large-scale production of human immunoglobulins in chicken eggs.

Example 2

Preparation Of Transgenic Chickens

Transgenic chickens which produce humanized antibodies may be prepared. To produce the transgenic chicken, Stage X (40b) embryos will be obtained from unincubated eggs laid by Barred Plymouth Rock hens. Blastodermal cells are harvested by enzymatic digestion of the intercellular matrix and DNA is introduced into the dispersed cells using lipofection reagents as described by Brazolot et al. and Fraser et al. The dispersed cells will then be injected into irradiated stage X recipient

embryos in eggs laid by White Leghorn hens as described by Carscience et al. On the fourth day after injection, the injected embryos are transferred to a surrogate shell (109, 109b) which increases the rate of hatching from approximately 10% of injected embryos to approximately 40% of injected embryos (Cochran and Etches, unpublished). At hatch, chimeras can be recognized by the presence of black down of donor (Barred Plymouth Rock) origin and yellow (White Leghorn) down of recipient origin. Hatchlings that show no evidence of incorporation of donor cells are discarded. Comb tissue and blood will be removed on the day of hatch and at 4 weeks of age respectively, and the presence of the DNA sequence coding for the production of the chimeric antibody will be determined by PCR. The presence of chimeric Ig will be assessed by ELISA conducted on the blood sample taken at 4 weeks of age. Chicks that carry the transgene will be grown to sexual maturity. The deposition of the chimeric Ab in developing ova will be assessed by ELISA conducted on extracts from yolks of eggs laid by female chimeras. Both male and female chimeras will be mated and the resulting offspring will be screened by PCR to identify those that contain the construct. It should be noted that the goal of producing chimeric antibody in eggs will be achieved in chimeras if transfected cells colonize the lymphoid system. A strain of chickens in which the DNA sequences encoding the production of chimeric antibody is incorporated as a Mendelian trait will be derived if the construct is incorporated into the germline. However, even in the absence of germline transmission we will gain significant new information about antibody production in chickens.

While the present invention has been described with reference to what are presently considered to be the preferred examples, it is to be understood that the invention is not limited to the disclosed examples. To the contrary, the invention is intended to cover various modifications and equivalent arrangements included within the spirit and scope of the appended claims.

All publications, patents and patent applications are herein incorporated by reference in their entirety to the same extent as if each

individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference in its entirety.

Table 1

Standard Conc. ng/ml	Log 10 of Standard Conc.	Abs. @ 492 nm of Standard Conc.	Average corr. for (-) control		Abs. @ 492nm for (-)control	Average (-) control	
0.098	-1.00877	0.079	0.085	-0.008	0.1	0.08	0.09
0.195	-0.70997	0.101	0.109	0.015			0.09
0.39	-0.40894	0.137	0.167	0.062			0.09
0.78	-0.10791	0.207	0.198	0.1125			0.09
1.56	0.193125	0.334	0.324	0.239			0.09
3.12	0.494155	0.637	0.578	0.5175			0.09
6.25	0.79588	0.959	1.017	0.898			0.09
12.5	1.09691	1.329	1.422	1.2855			0.09
25	1.39794	1.499	1.393	1.356			0.09

Table 2

Concentration of human immunoglobulin in yolk from hen #9185 (Cage #2)

Day	Concentration of hIg (ng/ml yolk)
1	undetectable
2	undetectable
3	undetectable
4	undetectable
5	undetectable
6	undetectable
7	undetectable
8	undetectable
9	undetectable
10	undetectable
11	undetectable
12	no egg
13	6.27
14	no egg
15	3.46
16	no egg
17	undetectable
18	undetectable
19	undetectable
20	undetectable
21	undetectable
22	undetectable
23	undetectable
24	undetectable
25	undetectable
26	undetectable
27	undetectable

We Claim:

1. An expression vector for delivering a recombinant protein to an egg comprising (i) a first DNA sequence encoding the recombinant protein (ii) a regulatory region sufficient to provide for expression of the protein and (iii) a second DNA sequence which can facilitate the delivery of the protein to an egg of an animal.

2. A vector according to claim 1 wherein the second DNA sequence encodes a protein or peptide which can bind to an egg.

3. A vector according to claim 2 wherein the second DNA encodes a portion of an immunoglobulin that can bind to the egg.

4. An expression vector for delivering a recombinant antibody to an egg comprising (i) a first DNA sequence encoding an immunoglobulin constant region (ii) a second DNA sequence encoding an immunoglobulin variable region and (iii) a regulatory region sufficient to provide for expression of the antibody.

5. A vector according to claim 4 wherein the constant region is derived from a human immunoglobulin gene.

6. A method of preparing a recombinant protein in an egg comprising:

a) introducing an expression vector into an egg-laying animal, wherein the expression vector comprises (i) a DNA sequence encoding the recombinant protein (ii) a regulatory region sufficient to provide for expression of the protein and (iii) A DNA sequence which facilitates the delivery of the protein to an egg of the animal;

b) allowing the animal to lay an egg;

c) obtaining the egg containing the recombinant protein; and optionally

d) isolating the recombinant protein from the egg.

7. A method of preparing a recombinant antibody in an egg comprising:

a) introducing an expression vector into an egg-laying animal, wherein the expression vector comprises (i) a first DNA sequence encoding an immunoglobulin constant region (ii) a second DNA sequence encoding an immunoglobulin variable region and (iii) a regulatory region sufficient to provide for expression of the antibody;

b) allowing the animal to lay an egg;

c) obtaining the egg containing the recombinant antibody; and optionally

d) isolating the recombinant protein from the egg.

8. A method of preparing a recombinant protein in an egg comprising:

a) introducing a transformed avian cell line that secretes a recombinant protein into an egg-laying animal wherein the avian cell line has been transformed with an expression vector comprising (i) a first DNA sequence encoding the recombinant protein (ii) a regulatory region sufficient to provide for expression of the protein and (iii) a second DNA sequence which can effect the delivery of the protein to an egg of an animal;

b) allowing the animal to lay an egg;

c) obtaining the egg containing the recombinant protein; and optionally

d) isolating the recombinant protein from the egg.

9. A method of preparing a recombinant antibody in a fowl egg comprising:

a) introducing a transformed avian cell line that secretes a recombinant antibody into an egg laying fowl wherein the avian cell line has been transformed with an expression vector comprising (i) a first

DNA sequence encoding an immunoglobulin constant region (ii) a second DNA sequence encoding an immunoglobulin variable region and (iii) a regulatory region sufficient to provide for expression of the antibody;

b) allowing the fowl to lay an egg;

c) obtaining the egg containing the recombinant antibody; and optionally

d) isolating the recombinant antibody from the egg.

10. A method of preparing an egg that is free of a particular pathogen comprising:

(a) introducing an antibody specific for the pathogen into an egg laying animal; and

(b) allowing the animal to lay an egg wherein the egg is substantially free of the pathogen.

11. An egg containing a recombinant protein.

12. An egg containing a recombinant antibody.

13. A method of immunizing an animal comprising administering a therapeutically effective amount of an egg according to claim 12.

14. A method according to claim 13 wherein said egg is administered orally.

15. A transformed avian cell line that secretes a recombinant antibody.

16. A transgenic avian animal whose germ line cells and somatic cells contain a recombinant gene encoding a recombinant antibody.

17. A method of preparing a transgenic avian animal that produces recombinant antibody comprising:

(i) inserting a recombinant expression vector into an avian embryo wherein said expression vector comprises (a) a DNA sequence encoding a constant region; (b) a DNA sequence encoding a desired variable region; and (c) regulatory sequences suitable for expression of the antibody in the avian cell; and

(ii) allowing the embryo to grow to maturity.

18. A method according to claim 17 wherein the constant region is derived from a human gene.

ABSTRACT OF THE DISCLOSURE

Methods for preparing recombinant proteins, such as humanized antibodies, in eggs and pharmaceutical compositions containing the recombinant proteins are disclosed.